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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/10, C12P 19/18

A1

(11) International Publication Number: **WO 99/15633**

(43) International Publication Date: 1 April 1999 (01.04.99)

(21) International Application Number: PCT/DK98/00412

(22) International Filing Date: 23 September 1998 (23.09.98)

(30) Priority Data:

1098/97	24 September 1997 (24.09.97)	DK
60/062,659	8 October 1997 (08.10.97)	US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: NOVEL CYCLOMALTODEXTRIN GLUCANOTRANSFERASE VARIANTS

(57) Abstract

The present invention relates to variants of cyclomaltodextrin glucanotransferase of increased product specificity.

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NOVEL CYCLOMALTODEXTRIN GLUCANOTRANSFERASE VARIANTS

TECHNICAL FIELD

The present invention relates to variants of cyclomaltodextrin glucanotransferase of increased product specificity.

BACKGROUND ART

Cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19), also designated cyclodextrin glucanotransferase or cyclodextrin glycosyltransferase, in the following termed CGTase, catalyses the conversion of starch and similar substrates into cyclomaltodextrins via an intramolecular transglycosylation reaction, thereby forming cyclomaltodextrins, in the following termed cyclodextrins (or CD), of various sizes. Commercially most important are cyclodextrins of 6, 7 and 8 glucose units, which are termed α-, β- and γ-cyclodextrins, respectively. Commercially less important are cyclodextrins of 9, 10, and 11 glucose units, which are termed δ-, ε-, and ζ-cyclodextrins, respectively.

Cyclodextrins are thus cyclic glucose oligomers with a hydrophobic internal cavity. They are able to form inclusion complexes with many small hydrophobic molecules in aqueous solutions, resulting in changes in physical properties, e.g. increased solubility and stability and decreased chemical reactivity and volatility. Cyclodextrins find applications particularly in the food, cosmetic, chemical and pharmaceutical industries.

Most CGTases have both starch-degrading activity and transglycosylation activity. Although some CGTases produce mainly α-cyclodextrins and some CGTases produce mainly β-cyclodextrins, CGTases usually form a mixture of α-, β- and γ-cyclodextrins. Selective precipitation steps with organic solvents may be used for the isolation of separate α-, β- and γ-cyclodextrins. To

avoid expensive and environmentally harmful procedures, the availability of CGTases capable of producing an increased ratio of one particular type of cyclodextrin, in particular with respect to α-, β- or γ-cyclodextrin, is desirable.

WO 96/33267 (Novo Nordisk) describes CGTase variants showing a modified substrate binding and/or product selectivity. Although CGTase variants produced by mutation at positions 47, 145, 146, 147, 196 or 371 have been described, the specific CGTase variants of this invention have never been described or even suggested.

SUMMARY OF THE INVENTION

The present invention provided novel CGTase variants of increased product specificity. Although CGTase variants of increased product specificity have been described in the prior art (WO 96/33267), the CGTase variants of the present invention have never been described or suggested.

Among the tremendous number of possible CGTase variants we have now succeeded in finding a limited number of variants showing increased product specificity when compared to the wild-type enzyme.

Accordingly the invention provides a CGTase variant of increased product specificity, in which one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47K; 47N; 47P; 47R; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145H; 145I; 145N; 145Q; or 145V;

(iii) Position 146: 146H, 146K; 146L; 146T; 146V; or 146Y;

(iv) Position 147: 147C; 147D; 147E; 147N; 147Q;

(v) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; or 196W; 196Y and/or

(vi) Position 371: 371C; 371E; 371F; 371H; 371I; 371K; 371L; 371M; 371Q; 371R; 371T; 371V; or 371W.

Amino Acids

In the context of this invention the following symbols and abbreviations for amino acids and amino acid residues are used:

A	=	Ala =	Alanine
C	=	Cys =	Cysteine
D	=	Asp =	Aspartic acid
E	=	Glu =	Glutamic acid
F	=	Phe =	Phenylalanine
G	=	Gly =	Glycine
H	=	His =	Histidine
I	=	Ile =	Isoleucine
K	=	Lys =	Lysine
L	=	Leu =	Leucine
M	=	Met =	Methionine
N	=	Asn =	Asparagine
P	=	Pro =	Proline
Q	=	Gln =	Glutamine
R	=	Arg =	Arginine
S	=	Ser =	Serine
T	=	Thr =	Threonine
V	=	Val =	Valine
W	=	Trp =	Tryptophan
Y	=	Tyr =	Tyrosine
B	=	Asx =	Asp or Asn
Z	=	Glx =	Glu or Gln
X	=	Xaa =	Any amino acid
*	=	Deletion or absent amino acid	

CGTase Variants

A CGTase variant of this invention is a CGTase variant or mutated CGTase, having an amino acid sequence not found in nature.

A CGTase variant or mutated CGTase of this invention may be considered a functional derivative of a precursor CGTase enzyme (i.e. the native, parental, or wild-type enzyme), and may be obtained by alteration of a DNA nucleotide sequence of a precursor gene or its derivatives, encoding the precursor enzyme.

The CGTase variant or mutated CGTase may be expressed and produced when the DNA nucleotide sequence encoding the CGTase variant is inserted into a suitable vector in a suitable host organism. The host organism is not necessarily identical to the organism from which the precursor gene originated.

In the literature, enzyme variants have also been referred to as mutants or muteins.

CGTase Numbering

In the context of this invention a specific numbering of amino acid residue positions in CGTase enzymes is employed. By alignment of the amino acid sequences of various known CGTases it is possible to unambiguously allot a CGTase amino acid position number to any amino acid residue position in any CGTase enzyme, which amino acid sequence is known.

Using the numbering system originating from the amino acid sequence of the CGTase obtained from *Bacillus circulans* Strain 251, aligned with the amino acid sequence of a number of other known CGTases, it is possible to indicate the position of an amino acid residue in a CGTase enzyme unambiguously.

This CGTase Numbering system has been described in WO 96/33267, see Table 1, pages 9-31 (in which table *Bacillus circulans* Strain 251 is represented as a). Table 1 of WO 96/33267 also shows the

protein sequences of a number of relevant CGTase and is hereby incorporated by reference.

In describing the various CGTase variants produced or contemplated according to the invention, the following nomenclatures are adapted for ease of reference:

[Original amino acid; Position; Substituted amino acid]

Accordingly, the substitution of serine with alanine in position 145 is designated as S145A.

Amino acid residues which represent insertions in relation to the amino acid sequence of the CGTase from *Bacillus circulans* Strain 251, are numbered by the addition of letters in alphabetical order to the preceding CGTase number, such as *e.g.* position 91aF for the "insert" Phe between Thr at position 91 and Gly at position 92 of the amino acid sequence of the CGTase from *Thermoanaerobacter sp.* ATCC 53627, cf. Table 1 (j).

Deletion of a proline at position 149 is indicated as P149*, and an insertion between position 147 and 148 where no amino acid residue is present, is indicated as *147aD for insertion of an aspartic acid in position 147a.

Multiple mutations are separated by slash marks ("/"), *e.g.* S145A/D147L, representing mutations in positions 145 and 147 substituting serine with alanine and aspartic acid with leucine, respectively.

If a substitution is made by mutation in *e.g.* a CGTase derived from a strain of *Bacillus circulans*, the product is designated *e.g.* "*B. circulans*/S145A".

All positions referred to in this application by CGTase numbering refer to the CGTase numbers described above.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is further illustrated by reference to the accompanying drawing, in which:

Figure 1 shows a cartoon of the substrate binding cleft of *B. circulans* st. 251 CGTase. Dote lines indicate hydrogen bonds between the enzyme (water) and the substrate. Cleavage point is defined between glucose residue -1 and 1.

Figure 2, 3 and 4 show cyclodextrins formed during incubation of (mutant) CGTase proteins from *Thermoanaerobacterium thermosulfurigenes*. A. wild-type (Figure 2); B. Variant D196H (Figure 3); C. variant D371R (Figure 4).

DETAILED DISCLOSURE OF THE INVENTION

The present invention provides novel CGTase variants, *i.e.* CGTase variants having an amino acid sequence not found in nature. Formally, the CGTase variant of the invention may be regarded as a functional derivative of a precursor CGTase enzyme (*i.e.* the native, parental, or wild-type enzyme), by substitution, insertion and/or deletion of one or more amino acid residue(s) of the precursor enzyme.

In the context of this invention, a CGTase variant of increased product specificity is a CGTase variant capable of producing an increased ratio of one particular type of cyclodextrin, when compared to the wild-type enzyme.

In a CGTase variant of the invention, one or more amino acid residues corresponding to the following positions (CGTase Numbering) have been introduced by substitution and/or insertion:

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47K; 47N; 47P; 47R; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145H; 145I; 145N; 145Q; or 145V;

(iii) Position 146: 146H, 146K; 146L; 146T; 146V; or 146Y;

(iv) Position 147: 147C; 147D; 147E; 147N; 147Q;

(v) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(vi) Position 371: 371C; 371E; 371F; 371H; 371I; 371K; 371L; 371M; 371Q; 371R; 371T; 371V; or 371W.

In a preferred embodiment, CGTase variants showing an increased product specificity with respect to the production of α-cyclodextrin are provided, in which variants one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47F; 47K; 47R; 47W; or 47Y;

(ii) Position 145: 145D; 145H; 145N; or 145Q;

(iii) Position 146: 146H, 146K; 146L; 146T; 146V; or 146Y;

(iv) Position 147: 147C; 147D; 147E; 147N; 147Q;

(v) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(vi) Position 371: 371C; 371H; 371K; 371R; or 371T.

In another preferred embodiment, CGTase variants showing an increased product specificity with respect to the production of β-cyclodextrin are provided, in which variants one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47N; 47P; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145I; 145N; or 145V;

(iii) Position 147: 147E;

(iv) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(v) Position 371: 371C; 371E; 371F; 371H; 371I; 371K; 371L; 371M; 371Q; 371R; 371T; 371V; or 371W.

In yet another preferred embodiment, CGTase variants showing an increased product specificity with respect to the production of γ-cyclodextrin are provided, in which variants one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47N; 47P; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145I; 145N; or 145V;

(iii) Position 147: 147E;

(iv) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(v) Position 371: 371C; 371E; 371F; 371H; 371K; 371M; 371Q; 371R; 371T; or 371W.

The CGTase variant of the invention may be derived from any CGTase enzyme found in nature. However, the CGTase variant of the invention preferably is derived from a microbial enzyme, preferably a bacterial enzyme, and preferably the CGTase variant is derived from a strain of *Bacillus*, a strain of *Brevibacterium*, a strain of *Clostridium*, a strain of *Corynebacterium*, a strain of *Klebsiella*, a strain of *Micrococcus*, a strain of *Thermoanaerobium*, a strain of *Thermoanaerobacter*, a strain of

Thermoanaerobacterium, a strain of *Thermoanaerobacterium*, or a strain of *Thermoactinomyces*.

In a more preferred embodiment, the CGTase variant of the invention is derived from a strain of *Bacillus autolyticus*, a strain of *Bacillus cereus*, a strain of *Bacillus circulans*, a strain of *Bacillus circulans* var. *alkalophilus*, a strain of *Bacillus coagulans*, a strain of *Bacillus firmus*, a strain of *Bacillus halophilus*, a strain of *Bacillus macerans*, a strain of *Bacillus megaterium*, a strain of *Bacillus ohbensis*, a strain of *Bacillus stearothermophilus*, a strain of *Bacillus subtilis*, a strain of *Klebsiella pneumonia*, a strain of *Thermoanaerobacter ethanolicus*, a strain of *Thermoanaerobacter finnii*, a strain of *Clostridium thermoamylolyticum*, a strain of *Clostridium thermosaccharolyticum*, or a strain of *Thermoanaerobacterium thermosulfurigenes*.

In a most preferred embodiment, the CGTase variant of the invention is derived from the strain *Bacillus sp.* Strain 1011, the strain *Bacillus sp.* Strain 38-2, the strain *Bacillus sp.* Strain 17-1, the strain *Bacillus sp.* 1-1, the strain *Bacillus sp.* Strain B1018, the strain *Bacillus circulans* Strain 8, the strain *Bacillus circulans* Strain 251, or the strain *Thermoanaerobacter* sp. ATCC 53627, or mutants or variants thereof.

If the CGTase variant of the invention is derived from a strain of *Bacillus circulans*, one or more of the amino acid residues corresponding to the following positions may be introduced:

(i) Position R47: R47C; R47D; R47E; R47F; R47G; R47I; R47K; R47N; R47P; R47S; R47T; R47V; R47W; or R47Y;

(ii) Position S145: S145D; S145H; S145I; S145N; S145Q; or S145V;

(iii) Position S146: S146H, S146K; S146L; S146T; S146V; or S146Y;

(iv) Position D147: D147C; D147E; D147N; D147Q;

(v) Position D196: D196C; D196E; D196F; D196G; D196H; D196I; D196K; D196L; D196M; D196P; D196Q; D196R; D196T; D196V; D196W; or D196Y and/or

(vi) Position D371; D371C; D371E; D371F; D371H; D371I; D371K; D371L; D371M; D371Q; D371R; D371T; D371V; or D371W.

Preferably the CGTase variant is derived from *Bacillus circulans* Strain 251, or a mutant or a variant thereof.

If the CGTase variant is derived from a strain of *Thermoanaerobacter sp.*, one or more of the amino acid residues corresponding to the following positions may be introduced:

(i) Position K47; K47C; K47D; K47E; K47F; K47G; K47I; K47N; K47P; K47R; K47S; K47T; K47V; K47W; or K47Y;

(ii) Position S145: S145D; S145H; S145I; S145N; S145Q; or S145V;

(iii) Position E146: E146H, E146K; E146L; E146T; E146V; or E146Y;

(iv) Position T147: T147C; T147D; T147E; T147N; T147Q;

(v) Position D196: D196C; D196E; D196F; D196G; D196H; D196I; D196K; D196L; D196M; D196P; D196Q; D196R; D196T; D196V; D196W; or D196Y and/or

(vi) Position D371: D371C; D371E; D371F; D371H; D371I; D371K; D371L; D371M; D371Q; D371R; D371T; D371V; or D371W.

Preferably the CGTase variant is derived from the strain *Thermoanaerobacter sp.* ATCC 53627, or a mutant or a variant thereof.

Example 1 describes the construction of *T. thermosulfurigenes* CGTase variants Asp196His (D196H) and Asp371Arg (D371R) with modified product specificity, in which site-directed mutagenesis has lead to an altered number of hydrogen bonds in the subsite of the active site cleft. The variants are derived from a *Thermoanaerobacter thermosulfurigenes* EM1 CGTase (i.e. the wild-type), obtained as described by Haeckel and Bahl [Haeckel, K., and

Bahl, H. (1989) *FEMS Microbiol. Lett.* 60, 333-338 or Knegtel R.M.A., Wind R.D., Rozeboom H.J., Kalk K.H., Buitelaar R.M., Dijkhuizen L., Dijkstra B.W. J. Mol. Biol. 256:611-622 (1996)].

In another preferred embodiment, the CGTase variant of the invention comprises one or more of the following amino acid residues (CGTase Numbering):

(i) 47K/145E/146V/147N;

(ii) 47K/145E/146E/147N;

(iii) 47K/145D/146R/147D;

(iv) 47K/145D/146E/147D;

(v) 47K/145E/146V/147N/196H;

(vi) 47K/145E/146E/147N/196H;

(vii) 47K/145E/146V/147N/196H/371R;

(viii) 47K/145E/146E/147N/196H/371R;

(ix) 47K/145D/146R/147D/196H;

(x) 47K/145D/146E/147D/196H;

(xi) 47K/145D/146R/147D/196H/371R; and/or

(xii) 47K/145D/146R/147D/196H/371R.

(xiii) 47K/196H;

(xiv) 47R/196H

(xv) 145E/146V/147N;

(xvi) 145E/146E/147N;

(xvii) 145D/146R/147D;

(xviii) 145D/146E/147D;

(xix) 47K/371R;

(xx) 47R/371R;

If the CGTase variant is derived from a strain of *Bacillus circulans* one or more of the following amino acid residues may be introduced:

(i) R47K/S145E/S146V/D147N;

(ii) R47K/S145E/S146E/D147N;

(iii) R47K/S145D/S146R;

(iv) R47K/S145D/S146E;

(v) R47K/S145E/S146V/D147N/D196H;

(vi) R47K/S145E/S146E/D147N/D196H;

(vii) R47K/S145E/S146V/D147N/D196H/D371R;

(viii) R47K/S145E/S146E/D147N/D196H/D371R;

(ix) R47K/S145D/S146R/D196H;

(x) R47K/S145D/S146E/D196H;

(xi) R47K/S145D/S146R/D196H/D371R;

(xii) R47K/S145D/S146R/D196H/D371R.

(xiii) R47K/D196H;

(xiv) S145E/S146V/D147N;

(xv) S145E/S146E/D147N;

(xvi) S145D/S146R;

(xvii) S145D/S146E;

(xviii) R47K/D371R;

Preferably the CGTase variant is derived from *Bacillus circulans* Strain 251, or a mutant or a variant thereof.

If the CGTase variant is derived from a strain of *Thermoanaerobacter sp.*, one or more of the following amino acid residues may be introduced:

(i) S145E/E146V/T147N;

(ii) S145E/T147N;

(iii) S145D/E146R/T147D;

(iv) S145D/T147D;

(v) S145E/E146V/T147N/D196H;

(vi) S145E/T147N/D196H;

(vii) S145E/E146V/T147N/D196H/D371R;

(viii) S145E/T147N/D196H/D371R;

(ix) S145D/E146R/T147D/D196H;

(x) S145D/T147D/D196H;

(xi) S145D/E146R/T147D/D196H/D371R;

(xii) S145D/E146R/T147D/D196H/D371R.

(xiii) S145E/E146V/T147N;

(xiv) S145E/T147N;

(xv) S145D/E146R/T147D;

(xvi) S145D/T147D; and/or

(xvii) K47R/D371R;

(xviii) K47R/D196H

Preferably the CGTase variant is derived from the strain *Thermoanaerobacter sp.* ATCC 53627, or a mutant or a variant thereof.

In a further aspect the invention relates to the use of a CGTase variant of the invention for increasing the α or β or γ-cyclodextrin content of the final cyclodextrin product of cyclodextrins processes.

In a final aspect the invention relates to a method of increasing the product specificity with respect to the production of α or β or γ-cyclodextrins, wherein one or more amino acid residues, corresponding to the positions of the CGTase

variants of the invention mentioned above, have been introduced by substitution and/or insertion.

EXAMPLES

EXAMPLE 1

Construction of *T. thermosulfurigenes* CGTase variants with modified product specificity.

Bacterial strains, plasmids and growth conditions

Escherichia coli JM109 [*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17(rK-,mK+) *rel*A1 *sup*E44 (*lac-pro*AB) [F' *tra*D36 *pro*AB *lac*IqZ M15] (Yanish-Perron et al. 1985 *Gene* 33, 103-119) was used for recombinant DNA manipulations. *Escherichia coli* PC1990 (Lazzaroni and Portalier 1979 *FEMS Microbiol. Lett.* 5, 411-416), known to leak periplasmic proteins into the supernatant because of a mutation in its *tolB* locus, was used for production of CGTase (mutant) proteins. Plasmid pCT2, a derivative of pUC18 containing the *amyA* gene of *Thermoanaerobacterium thermosulfurigenes* EM1 (Knegtel R.M.A., Wind R.D., Rozeboom H.J., Kalk K.H., Buitelaar R.M., Dijkhuizen L., Dijkstra B.W. J. Mol. Biol. 256:611-622 (1996)), was used for site-directed mutagenesis, sequencing and expression of the CGTase (mutant) proteins. Plasmid-carrying bacterial strains were grown on LB medium in the presence of 100 µg/ml ampicillin. When appropriate, IPTG (isopropyl-ß-D-thiogalactopyranoside) was added at a concentration of 0.1 mM for induction of protein expression.

DNA manipulations

DNA manipulations and transformation of *E. coli* were essentially as described by Sambrook et al. [Sambrook, J., Fritsch, E.J.,

and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press, New York]. Transformation of *E. coli* by electroporation was performed using the Bio-Rad gene pulser apparatus (Bio-Rad, Veenendaal, The Netherlands). The selected conditions were 2.5 kV, 25 µF and 200 Ω.

Site-directed mutagenesis

Mutant CGTase genes were constructed via a double PCR method using the *Pfu* DNA polymerase from Stratagene (Westburg, Leusden, The Netherlands). A first PCR reaction was carried out with the mutagenesis primer for the coding strand plus a primer 195-715 bp downstream on the template strand. The reaction product was subsequently used as primer in a second PCR reaction together with a primer 295-815 bp upstream on the coding strand. The product of the last reaction was cut with *NcoI* and *MunI*, and exchanged with the corresponding fragment (900 bp) from the vector pCT2. The resulting (mutant) plasmid was transformed to *E. coli* JM109 for sequencing and *E. coli* PC1990 for production of the (mutant) proteins. The following oligonucleotides were used:

D196H 5'-CGTAACTTATTT**CAT**TTAGCAGATCTAAATCAACAG-3'
(SEQ ID No. 1)

D371R 5'-GACAGGCAATGGA**CGT**CCTTATAATAGAGC-3'
(SEQ ID NO. 2).

Successful mutations resulted in the underlined restriction sites (*BglII* for D196H and *AatII* for D371R), which allowed quick screening of transformants. Mutations were verified by DNA sequencing (Sanger et al. 1977 *Proc. Natl. Sci. USA* 74, 5463-5467). All 900 bp on the *MunI-NcoI* fragment obtained by PCR were checked by DNA-sequencing.

Production and purification of CGTase proteins

For production of CGTase proteins, *E. coli* PC1990 was grown in a 2-liter fermentor at pH 7.0 and 30 C. The medium contained 2% (w/w) tryptone (Oxoid, Boom BV, Meppel, The Netherlands), 1% (w/w) yeast extract (Oxoid), 1% (w/w) sodium chloride, 1% (w/w) casein hydrolysate (Merck, Darmstadt, Germany), 100 µg/l ampicillin and 0.1 mM IPTG. Growth was monitored by measuring the optical density at 450 nm. When the optical density at 450 nm reached 2 to 3, an extra amount of 50 g tryptone was added to the fermentor. Cells were harvested after 20-24 hours of growth (8,000 *g*, 30 minutes, 4°C), at an optical density of 8-12, and the supernatant was used for further purification of the CGTases. Supernatant was directly applied to an α-CD-sepharose-6FF affinity column (Monma et al. 1988 *Biotechnol. Bioeng.* 32, 404-407). After washing the column with 10 mM sodium acetate (pH 5.5), the CGTase was eluted with the same buffer supplemented with 1% (w/w) α-CD. Purity and molecular weight of the CGTase (mutant) proteins were checked on SDS-PAGE (Wind et al. 1995 *Appl. Environ. Microb.* 61, 1257-1265). Protein concentrations were determined by the method of Bradford, using the Coomassie protein assay reagent of Pierce (Pierce Europe bv, Oud-Beijerland, The Netherlands).

Enzyme assays

All assays were standardly performed at pH 6.0 and 50°C. Cyclization and saccharifying assays were performed as described by Penninga et al. (Penninga, D., Strokopytov, B., Rozeboom, H.J., Lawson, C.L., Dijkstra, B.W., Bergsma, J., and Dijkhuizen, L. (1995) *Biochemistry* 34, 3368-3376). Units for the different reactions were defined as the amount of enzyme producing 1 µmol of substrate at pH 6.0 and 50°C.

HPLC product analysis

Formation of cyclodextrins was measured under industrial process conditions by incubation of 0.1 U/ml CGTase (ß-CD forming activity) with 10% Paselli WA4 (pregelatinized drum-dried starch with a high degree of polymerization; AVEBE, The Netherlands) in 10 mM sodium citrate buffer (pH 6.0) at 60°C for 45 hours. Samples were taken at regular time intervals and boiled for 10 minutes. Products formed were analyzed by HPLC, using a 25-cm Econosil-NH2 10-µm column (Alltech Nederland bv, Breda, The Netherlands) eluted with acetonitrile-water (65:45) at 1 ml/minute. Products were detected by a refractive index detector (Waters 410, Waters Chromatography Division, Milford, USA). The temperature of the flow cell and column was set at 50°C, to avoid possible precipitation of starch. Formation of linear products was directly analyzed. Formation of CD's was analyzed after incubation of the samples with an appropriate amount of β-amylase (type I-B from Sweet potato, Sigma, Boom BV, Meppel, The Netherlands).

Cyclodextrin product specificity

In order to change the product specificity of *Thermoanaerobacterium* CGTase, we replaced Asp196 by His (D196H) and Asp371 by Arg (D371R). In mutant D196H production of α-CD was increased at the expense of the production of ß-CD when compared to the wild-type CGTase (Figure 2). The cyclodextrin product ratio was changed from 28:58:14 (α:ß:γ) for the wild-type CGTase to 35:49:16 for mutant D196H (Table 1). This is according to expectations on the basis of the structural work. By the replacement of Asp197 by His a larger residue is introduced, this would block the so-called straight substrate binding mode. Furthermore, His 197 is probably also able to form hydrogen bonds with the substrate bound in the 'bent' mode. These results lean more vigour to the

theory that the bent conformation is correlated with α-cyclodextrin production, and show that it can be used to rationally engineer a CGTase with desired product specificity.

Cyclization activity (U/ml	Product Ratio (%) of Starch α	β	γ	Conversion into cyclodextrins(%)
Wild-type	163	28	58	14	35
D196H	144	35	49	16	37
D371R	14	6	68	26	29

Table 1: Starch conversion of *T. thermosulfurigenes* wild-type and mutant CGTase proteins. Proteins (0.1 U/ml β-CD forming activity) were incubated for 45H at pH 6.0 and 60°C with 10% Paselli WA4.

Asp 371 has a very important role in substrate binding at subsite 2, both in the *BC251* and *Tabium* CGTase. Replacement of Asp371 by Arg resulted in the introduction of a very bulky amino acid, which would probably interfere severely with binding of all kinds of substrates at subsite 2, thereby explaining the overall decrease of activity. Apart from this low activity, the product ratio changed from 28:58:14 for the wild-type enzyme to 6:68:26 for mutant D371H (Table 1, Figure 2). This suggests that the cyclisation reaction leading to a α-cyclodextrin is more hampered by the Arg 371 than the other cyclisation reactions. Probably this bulky residue is sterically hindering the 'bent' conformation. These results show that the *Tabium* CGTase can changed from a α/β-cyclodextrin producer to a β/γ-cyclodextrin producer by just one mutation, illustrating the feasibility of CGTase protein engineering.

The invention is further illustrated with reference to the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

CLAIMS

1. A CGTase variant of increased product specificity, in which one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47K; 47N; 47P; 47R; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145H; 145I; 145N; 145Q; or 145V;

(iii) Position 146: 146H, 146K; 146L; 146T; 146V; or 146Y;

(iv) Position 147: 147C; 147D; 147E; 147N; 147Q;

(v) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(vi) Position 371: 371C; 371E; 371F; 371H; 371I; 371K; 371L; 371M; 371Q; 371R; 371T; 371V; or 371W.

2. The CGTase variant of claim 1, being K47R.

3. The CGTase variant of claim 1, being D196H.

4. The CGTase variant of claim 1, being D371R.

5. The CGTase variant according to claim 1, showing an increased product specificity with respect to the production of α-cyclodextrin, in which one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47F; 47K; 47R; 47W; or 47Y;

(ii) Position 145: 145D; 145H; 145N; or 145Q;

(iii) Position 146: 146H, 146K; 146L; 146T; 146V; or 146Y;

(iv) Position 147: 147C; 147D; 147E; 147N; 147Q;

(v) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; 196Y and/or

(vi) Position 371: 371C; 371H; 371K; 371R; or 371T.

6. The CGTase variant according to claim 1, showing an increased product specificity with respect to the production of β-cyclodextrin, in which one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47N; 47P; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145I; 145N; or 145V;

(iii) Position 147: 147E;

(iv) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(v) Position 371: 371C; 371E; 371F; 371H; 371I; 371K; 371L; 371M; 371Q; 371R; 371T; 371V; or 371W.

7. The CGTase variant according to claim 1, showing an increased product specificity with respect to the production of γ-cyclodextrin, in which one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position 47: 47C; 47D; 47E; 47F; 47G; 47I; 47N; 47P; 47S; 47T; 47V; 47W; or 47Y;

(ii) Position 145: 145D; 145I; 145N; or 145V;

(iii) Position 147: 147E;

(iv) Position 196: 196C; 196E; 196F; 196G; 196H; 196I; 196K; 196L; 196M; 196P; 196Q; 196R; 196T; 196V; 196W; or 196Y and/or

(v) Position 371: 371C; 371E; 371F; 371H; 371K; 371M; 371Q; 371R; 371T; or 371W.

8. The CGTase variant according to any of claims 1-7, which is derived from a strain of *Bacillus*, a strain of *Brevibacterium*, a strain of *Clostridium*, a strain of *Corynebacterium*, a strain of *Klebsiella*, a strain of *Micrococcus*, a strain of *Thermoanaerobium*, a strain of *Thermoanaerobacterium*, a strain of *Thermoanaerobacter*, or a strain of *Thermoactinomyces*.

9. The CGTase variant according to claim 8, which is derived from a strain of *Bacillus autolyticus*, a strain of *Bacillus cereus*, a strain of *Bacillus circulans*, a strain of *Bacillus circulans* var. *alkalophilus*, a strain of *Bacillus coagulans*, a strain of *Bacillus firmus*, a strain of *Bacillus halophilus*, a strain of *Bacillus macerans*, a strain of *Bacillus megaterium*, a strain of *Bacillus ohbensis*, a strain of *Bacillus stearothermophilus*, or a strain of *Bacillus subtilis*.

10. The CGTase variant according to claim 9, which is derived from the strain *Bacillus sp.* Strain 1011, the strain *Bacillus sp.* Strain 38-2, the strain *Bacillus sp.* Strain 17-1, the strain *Bacillus sp.* 1-1, the strain *Bacillus sp.* Strain B1018, the strain *Bacillus circulans* Strain 8, or the strain *Bacillus circulans* Strain 251, or a mutant or a variant thereof.

11. The CGTase variant according to claim 10, which is derived from the strain *Bacillus circulans* Strain 251, or a mutant of a variant thereof.

12. The CGTase variant according to claim 8, which is derived from a strain of *Klebsiella pneumonia*, a strain of *Thermoanaerobacter ethanolicus*, a strain of *Thermoanaerobacter finnii*, a strain of *Clostridium thermoamylolyticum*, a strain of

Clostridium thermosaccharolyticum, or a strain of *Thermoanaerobacterium thermosulfurigenes*.

13. The CGTase variant according to claim 8, which is derived from the strain *Thermoanaerobacter sp.* ATCC 53627, or a mutant or a variant thereof.

14. The CGTase variant according to any of claims 1-7, which is derived from a strain of *Bacillus circulans*, and in which variant one or more of the amino acid residues corresponding to the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position R47: R47C; R47D; R47E; R47F; R47G; R47I; R47K; R47N; R47P; R47S; R47T; R47V; R47W; or R47Y;

(ii) Position S145: S145D; S145H; S145I; S145N; S145Q; or S145V;

(iii) Position S146: S146H, S146K; S146L; S146T; S146V; or S146Y;

(iv) Position D147: D147C; D147E; D147N; D147Q;

(v) Position D196: D196C; D196E; D196F; D196G; D196H; D196I; D196K; D196L; D196M; D196P; D196Q; D196R; D196T; D196V; D196W; or D196Y and/or

(vi) Position D371: D371C; D371E; D371F; D371H; D371I; D371K; D371L; D371M; D371Q; D371R; D371T; D371V; or D371W.

15. The CGTase variant according to claim 11 which is derived from *Bacillus circulans* Strain 251, or a mutant or a variant thereof.

16. The CGTase variant according to any of claims 1-7, which is derived from a strain of *Thermoanaerobacter sp.*, and in which variant one or more of the amino acid residues corresponding to

the following positions have been introduced by substitution and/or insertion (CGTase Numbering):

(i) Position K47: K47C; K47D; K47E; K47F; K47G; K47I; K47N; K47P; K47R; K47S; K47T; K47V; K47W; or K47Y;

(ii) Position S145: S145D; S145H; S145I; S145N; S145Q; or S145V;

(iii) Position E146: E146H, E146K; E146L; E146T; E146V; or E146Y;

(iv) Position T147: T147C; T147D; T147E; T147N; T147Q;

(v) Position D196: D196C; D196E; D196F; D196G; D196H; D196I; D196K; D196L; D196M; D196P; D196Q; D196R; D196T; D196V; D196W; D196Y and/or

(vi) Position D371: D371C; D371E; D371F; D371H; D371I; D371K; D371L; D371M; D371Q; D371R; D371T; D371V; or D371W.

17. The CGTase variant according to claim 16, which is derived from the strain *Thermoanaerobacter sp.* ATCC 53627, or a mutant or a variant thereof.

18. The CGTase variant according to any of claims 1-13, which comprises one or more of the following amino acid residues (CGTase Numbering):

(i) 47K/145E/146V/147N;

(ii) 47K/145E/146E/147N;

(iii) 47K/145D/146R/147D;

(iv) 47K/145D/146E/147D;

(v) 47K/145E/146V/147N/196H;

(vi) 47K/145E/146E/147N/196H;

(vii) 47K/145E/146V/147N/196H/371R;

(viii) 47K/145E/146E/147N/196H/371R;

(ix) 47K/145D/146R/147D/196H;

(x) 47K/145D/146E/147D/196H;

(xi) 47K/145D/146R/147D/196H/371R;

(xii) 47K/145D/146R/147D/196H/371R.

(xiii) 47K/196H;

(xiv) 47R/196H;

(xv) 145E/146V/147N;

(xvi) 145E/146E/147N;

(xvii) 145D/146R/147D;

(xviii) 145D/146E/147D; and/or

(xix) 47K/371R;

(xx) 47R/371R;

19. The CGTase variant according to claim 18, which is derived from a strain of *Bacillus circulans*, and which comprises one or more of the following amino acid residues (CGTase Numbering):

(i) R47K/S145E/S146V/D147N;

(ii) R47K/S145E/S146E/D147N;

(iii) R47K/S145D/S146R;

(iv) R47K/S145D/S146E;

(v) R47K/S145E/S146V/D147N/D196H;

(vi) R47K/S145E/S146E/D147N/D196H;

(vii) R47K/S145E/S146V/D147N/D196H/D371R;

(viii) R47K/S145E/S146E/D147N/D196H/D371R;

(ix) R47K/S145D/S146R/D196H;

(x) R47K/S145D/S146E/D196H;

(xi) R47K/S145D/S146R/D196H/D371R;

(xii) R47K/S145D/S146R/D196H/D371R.

(xiii) R47K/D196H;

(xiv) S145E/S146V/D147N;

(xv) S145E/S146E/D147N;

(xvi) S145D/S146R;

(xvii) S145D/S146E;

(xviii) R47K/D371R;

20. The CGTase variant according to claim 19, which is derived from *Bacillus circulans* Strain 251, or a mutant or a variant thereof.

21. The CGTase variant according to claim 18, which is derived from a strain of *Thermoanaerobacter sp.*, and which comprises one or more of the following amino acid residues (CGTase Numbering):

(i) S145E/E146V/T147N;

(ii) S145E/T147N;

(iii) S145D/E146R/T147D;

(iv) S145D/T147D;

(v) S145E/E146V/T147N/D196H;

(vi) S145E/T147N/D196H;

(vii) S145E/E146V/T147N/D196H/D371R;

(viii) S145E/T147N/D196H/D371R;

(ix) S145D/E146R/T147D/D196H;

(x) S145D/T147D/D196H;

(xi) S145D/E146R/T147D/D196H/D371R;

(xii) S145D/E146R/T147D/D196H/D371R.

(xiii) S145E/E146V/T147N;

(xiv) S145E/T147N;

(xv) S145D/E146R/T147D;

(xvi) S145D/T147D; and/or

(xvii) K47R/D371R;

(xviii) K47R/D196H

22. The CGTase variant according to claim 21, which is derived from the strain *Thermoanaerobacter sp.* ATCC 53627, or a mutant or a variant thereof.

23. Use of a CGTase variant according to any of claims 1-22 for increasing the α-cyclodextrin content of the final cyclodextrin product of cyclodextrins processes.

24. Use of a CGTase variant according to any of claims 1-22 for increasing the β-cyclodextrin content of the final cyclodextrin product of cyclodextrins processes.

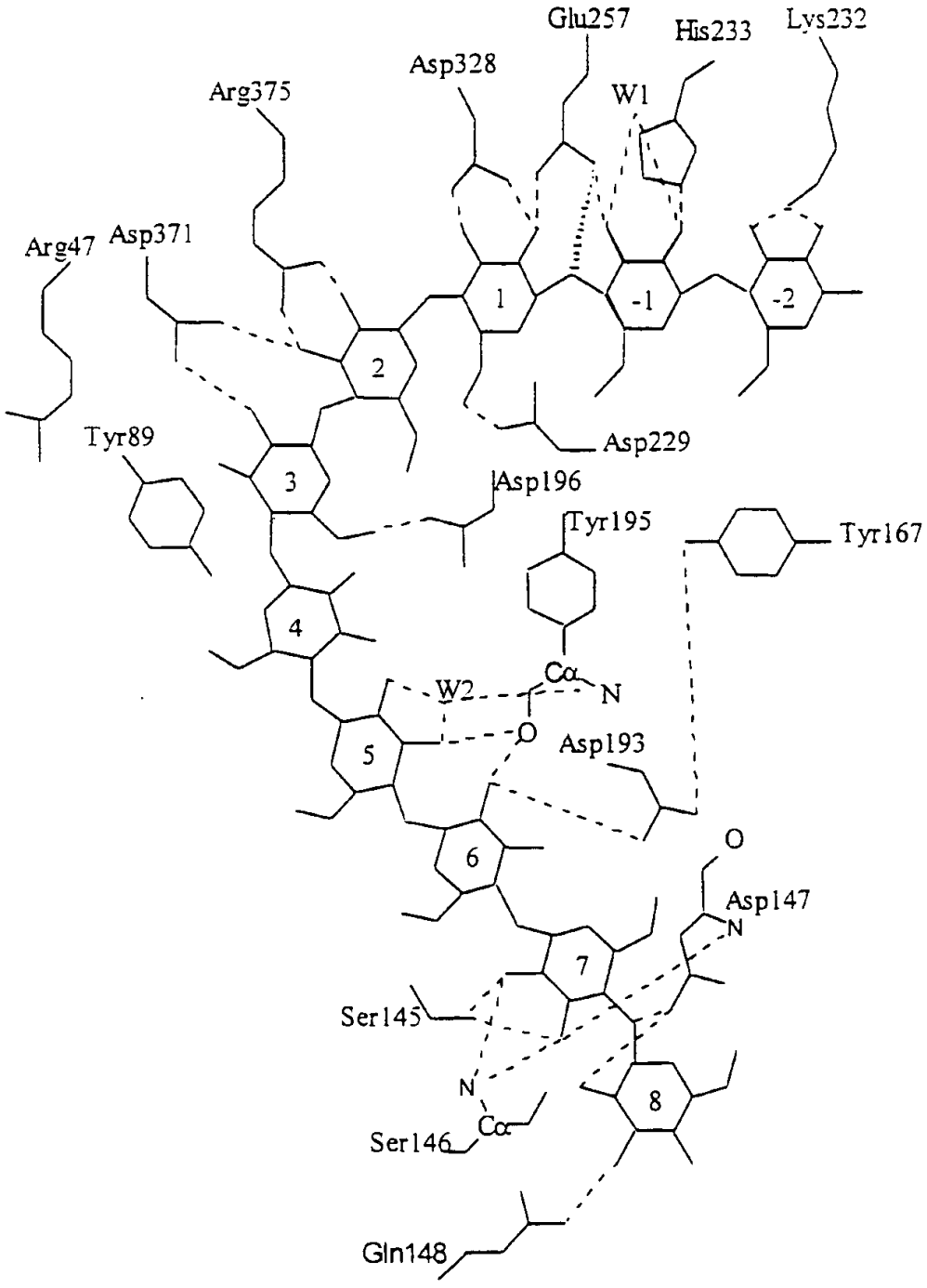
25. Use of a CGTase variant according to any of claims 1-22 for increasing the γ-cyclodextrin content of the final cyclodextrin product of cyclodextrins processes.

26. A method of increasing the product specificity with respect to the production of α-cyclodextrins, wherein one or more amino acid residues corresponding to the positions of the variants of any of claims 1-22 have been introduced by substitution and/or insertion.

27. A method of increasing the product specificity with respect to the production of β-cyclodextrins, wherein one or more of the amino acid residues corresponding to the positions of the variant of any of claims 1-22 have been introduced by substitution and/or insertion.

28. A method of increasing the product specificity with respect to the production of γ-cyclodextrins, wherein one or more of the amino acid residues corresponding to the positions of the variant

of any of claims 1-22 have been introduced by substitution and/or insertion.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Tyr195
Tyr167
4
Cα
W2
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Asp193
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Ser145
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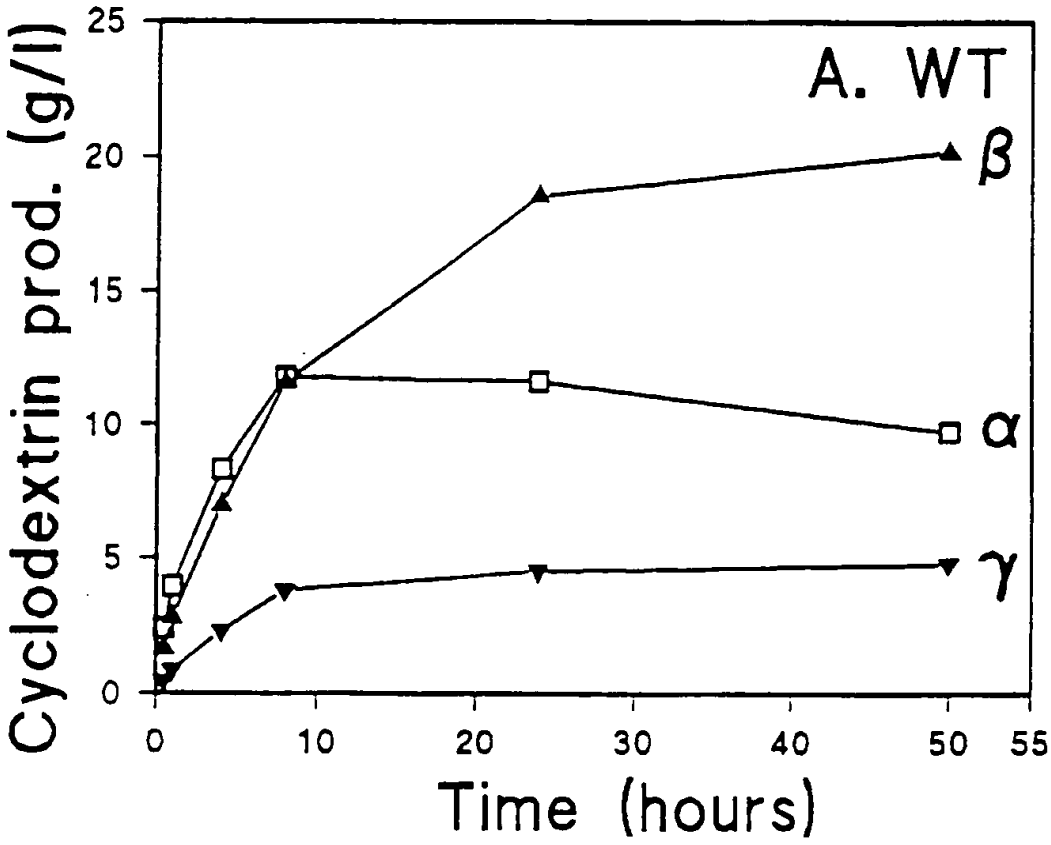
Cα
Ser146
Gln148

Fig. 1


A. WT
β
α
γ
Cyclodextrin prod. (g/l)
Time (hours)
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10
15
20
25
0
10
20
30
40
50
55

Fig. 2

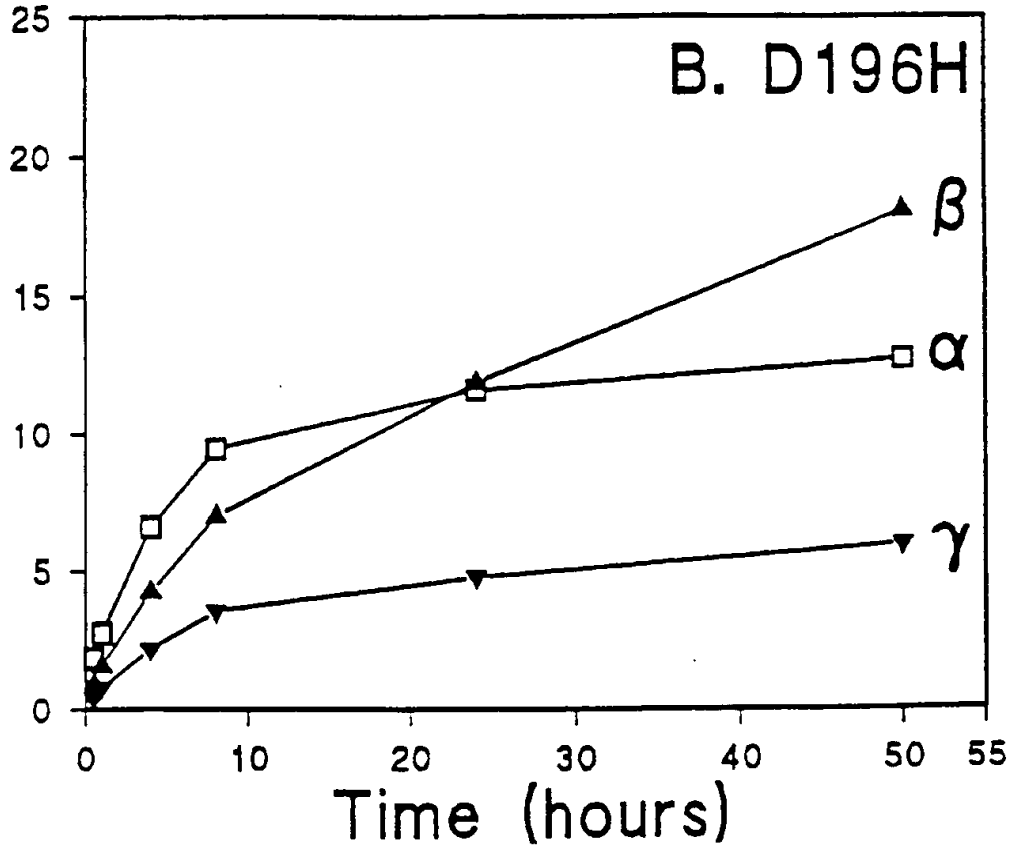

Fig. 3

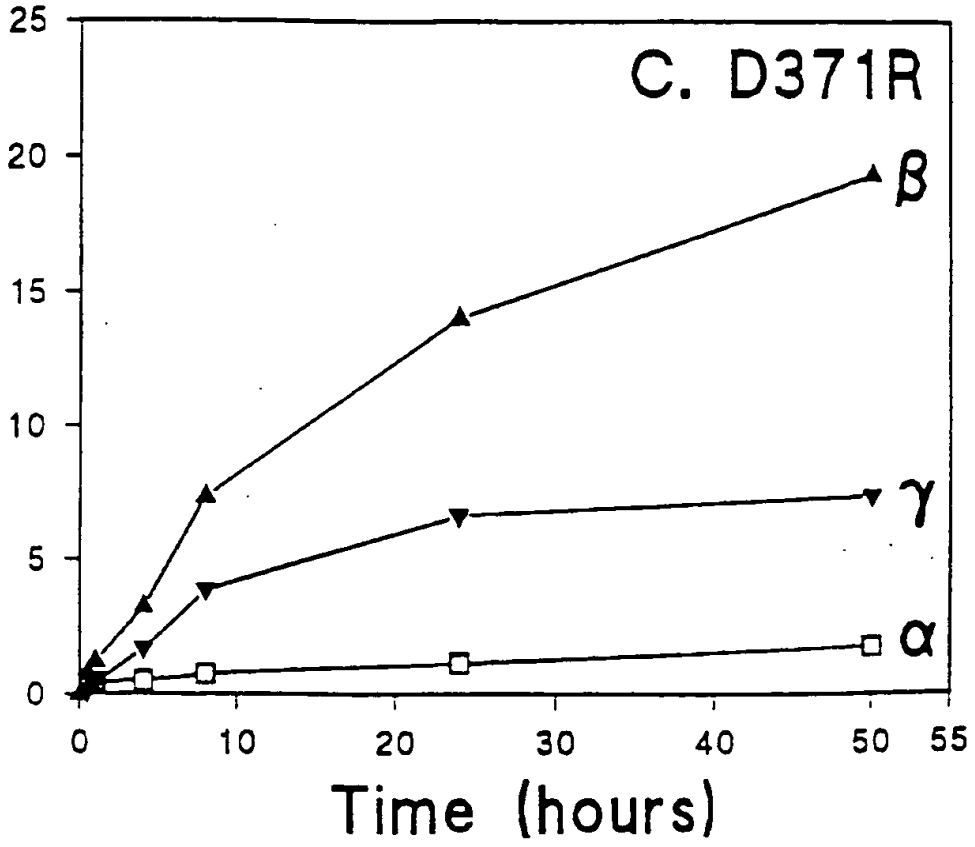

Fig. 4

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK 2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(ii) TITLE OF INVENTION: Novel CGTase variants
(iii) NUMBER OF SEQUENCES: 2
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

2) INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) OTHER INFORMATION: /desc: "D196H oligo"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGTAACTTAT TTCATTTAGC AGATCTAAAT CAACAG 36

2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) OTHER INFORMATION: /desc: "D371R oligo"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GACAGGCAAT GGACGTCCTT ATAATAGAGC 30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00412

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/10, C12P 19/18
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, BIOSIS, EMBASE, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9633267 A1 (NOVO NORDISK A/S), 24 October 1996 (24.10.96), see claims 45, 54-58, 65, 72 --	1-28
A	EP 0802259 A1 (CONSORTIUM FüR ELEKTROCHEMISCHE INDUSTRIE GMBH), 22 October 1997 (22.10.97) --	1-28
A	Patent Abstracts of Japan, & JP,A,5244945, (NIPPON SHOKUHIN KAKO CO LTD) 24 September 1993 --	1-28

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 January 1999	13 -01- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00412

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	File WPI, Derwent accession no. 93-308317, Uozumi T: "Glucano:transferase having increased relative prodn. of gamma- to beta-cyclodextrin - consists of modified aminoacid sequence of Bacillus ohbensis and is prepd. by culturing microbe transformed by gene coding the enzyme"; & JP,A,5219948, 930831 --	1-28
A	File WPI, Derwent accession no. 93-347473, NIPPON SHOKUHIN KAKO KK: "New alpha-cyclodextrin prepn. - by substituting tyrosine for phenyl alanine of cyclo malto-dextrin glucano-transfe-rase"; & JP,A,5244945, 930924 --	1-28
A	File WPI, Derwent accession no. 95-100943, OJI CORN STARCH CO LTD: "Modified cyclo-malto-dextrin glucano-transferase and corresp. gene - useful in the prodn. of gamma-dextrin"; & JP,A,7023781, 950127 --	1-28
A	File WPI, Derwent accession no. 93-103608, OJI CORN STARCH CO LTD: "Mutated cyclo malto dextran glucan transferase CGTase - prepd. by culturing transformants contg. CGTase expression vector using starch-contg. medium and sepq. gamma cyclodextrin from mixt."; & JP,A,5041985, 930223 --	1-28
A	Databas Medline, accession no. 97217591; & Biochem Mol Biol Int, Vol. 41, häfte 2, February 1997, Kim YH et al: "Effect on product specificity of cyclodextrin glycosyltransferase by site-directed mutagenesis", page 227 - page 234 --	1-28

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00412

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Databas Medline, accession no. 94235226; & J. Biotechnol, vol. 28, häfte 3, 28 February 1994, Sin KA et al: "Replacement of an amino acid residue of cyclodextrin glucanotransferase of . Bacillus ohbensis doubles the production of gamma--cyclodextrin", page 283 - page 288 --	1-28
A	Databas Medline, accession no. 93054368; & J Bacteriol vol. 174, häfte 22, November 1992, Fujiwara S. et al: "Analysis of mutations in cyclodextrin glucanotransferase from Bacillus stearothermophilus which affect cyclization characteristics and thermostability", page 7478 - page 7481 -- --------	1-28

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 98/00412

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

The invention claimed relates to variants of cyclomaltodextrin glucanotransferase (CGTase) enzymes with increased product specificity.

Unity of invention exists only when there is a technicaal relationship among the claimed inventions involving one or more of the same or corresponding "special technical features"-i.e. features that define a contribution which each of the inventions make over prior art. (See Annex B to Administrative Instructions and Rule 13.1)

WO 96/33267 discloses variants of cyclomaltodextrin having increased product specificity due to modifications in the amino acid sequence. Among others the amino acids at positions 47,196 and 371 have been modified. Therefore the different variants claimed cannot be seen as a unifying novel special technical feature.

Accordingly , The claims consists of at least the following six inventions :

1) Claim2 and part of the other claims directed to variants of CGTase being modified at position 47.

2) Claim 3 and part of the other claims directed to variants of CGTase being modified at position 196.

3)Claim 4 and part of the other claims directed to variants of CGTase being modified at position 371.

4)Part of the claims directed to variants of CGTase being modified at position 145.

5) " 146.

6) " 147.

As all claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fee.

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/12/98

International application No.
PCT/DK 98/00412

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9633267 A1	24/10/96	AU 5396896 A	07/11/96
		CA 2217876 A	24/10/96
		EP 0822982 A	11/02/98
EP 0802259 A1	22/10/97	CA 2199904 A	18/10/97
		CZ 9700298 A	12/11/97
		DE 19615336 A	23/10/97
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		JP 10033187 A	10/02/98

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